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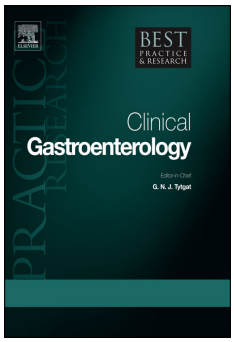

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Pathophysiology of Primary Biliary Cholangitis

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Abstract

Primary biliary cholangitis is a prototypical autoimmune disease characterized by an overwhelming female predominance, a distinct clinical phenotype, and disease specific anti-mitochondrial antibodies targeted against a well-defined auto-antigen. In a genetically susceptible host, multi-lineage loss of tolerance to the E2 component of the 2-oxo-dehydrogenase pathway and dysregulated immune pathways directed at biliary epithelial cells leads to cholestasis, progressive biliary fibrosis, and cirrhosis in a subset of patients. Several key insights have shed light on the complex pathogenesis of disease. First, characteristic anti-mitochondrial antibodies (AMAs) target lipoic acid containing immunodominant epitopes, particularly pyruvate dehydrogenase complex (PDC-E2), on the inner mitochondrial membrane of BECs. Next, breakdown of the protective apical bicarbonate rich umbrella may sensitize BECs to aberrant apoptotic pathways leaving the antigenic PDC-E2 epitope immunologically tact within an apoptotic bleb. A multi-lineage immune response ensues characterized by an imbalance between effector and regulatory activity resulting in progressive and self-perpetuating biliary injury. Genome wide studies shed light on important pathways involved in disease, key among them being IL-12. Epigenetic mechanisms and microRNAs may play help shed light on the missing heritability and female preponderance of disease. Taken together, these findings have dramatically advanced our understanding of disease and may lead to important therapeutic advances.

Keywords

Immunology, apoptosis, autoimmunity, cholangiocyte

INTRODUCTION

Primary Biliary Cholangitis (PBC) is an uncommon autoimmune liver disease characterized by progressive cholestasis, anti-mitochondrial antibodies (AMA), and histologic features of lymphocytic cholangitis and ductopenia(1). Its female predominance, characteristic phenotype, and well-defined autoantigen make it an archetypal autoimmune disease(2). Dysregulation of the innate and adaptive arms of the immune system occurs as a result of a distinct loss of tolerance to the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC-E2) resulting in a targeted immune response directed at biliary epithelial cells (BEC) (3). Disease specific AMAs are a hallmark of the disease and despite the ubiquitous nature of this autoantigen the inflammatory response in PBC is limited to the biliary epithelia(4).

Genetic studies have highlighted the importance of immune regulation in the pathogenesis of PBC and have identified aberrant pathways involved in antigen presentation, T and myeloid cell differentiation, and B cell function as contributing to disease(5). The relevance of the IL-12 signalling axis in PBC was emphasized in genome wide association surveys (GWAS) and reinforced by animal models(6, 7). More recently, a link between biliary injury, immune activation, and epigenetic regulation has been suggested which implicates loss of the protective biliary bicarbonate umbrella in biliary injury via downregulation of the $Cl^-/HCO3^-$ exchanger AE2 (anion exchanger 2)(8) Its seems plausible that on a permissive genetic background, exposure to a putative molecular mimic triggers a multi-lineage immune response targeted at BECs. Injured BEC's with dysfunctional AE2 are sensitized to apoptosis leading to exposed PDC-E2 within an apoptotic bleb that perpetuates focused biliary injury aggravated by an imbalance between effector and regulatory immune activity, ultimately leading to progressive cholestasis and fibrosis (Figure 1).

In this review we aim to provide an overview of the mechanisms contributing to the pathogenesis of PBC. A clear understanding of the biologic basis of disease is essential if progress is going to be made to expand the therapeutic options available to these patients with the aim of improving clinical outcomes.

AUTOANTIBODIES

Antimitochondrial Antibody

The AMA response is considered the most highly directed autoimmune marker in any disease, yet intriguingly, despite the presence of mitochondria in all cells, only the small bile ducts are targeted in PBC. AMA's are primarily targeted to the immunodominant PDC-E2 autoantigen on the 2-oxoacid dehydrogenase complexes (2-OADCs) located on the inner mitochondrial membrane(9). In addition to PDC-E2 other mitochondrial auto-antigens are also relevant including the E2 subunit of the 2-oxoglutarate dehydrogenase complex (OGDC-E2), the E2 subunit of the 2-oxoacid dehydrogenase complex (BCOADC-E2), and the E3-binding protein (E3BP)(10). Each complex has three subunits (E1-E3) though the E2 subunit remains most relevant particularly as it relates to its lipoyl domain which contains an essential lysine residue to which the lipoic acid cofactor is covalently attached(11, 12). All immunodominant epitopes contain a similar motif with the lipoic acid-lysine bond at position 173 being necessary for antigen recognition(13, 14) (Figure 2). T and B cell epitopes both include the lipoylated K173 amino acid of the inner domain(15) and PDC-E2 autoreactive CD4 and CD8 T cells are enriched in the liver and hilar lymph nodes in patients with both early and late stage disease (13, 16). While AMA's may not be independently pathogenic *in vivo*, in the presence of macrophages and BEC apotopes a burst of proinflammatory cytokines develops resulting in perpetual biliary inflammation(17).

Antinuclear Antibodies

Anti-nuclear antibodies (ANA's) are detected in 30-50% of patients with PBC and their presence can be important in diagnosis and prognosis(18). The pathogenic significance of ANAs in PBC is less clear, though PBC specific ANAs yield specific indirect immunofluorescence patterns based on their corresponding nuclear antigens (Table 1)(19). A nuclear rim pattern is observed with anti-gp210 ANA's that recognize antigenic epitopes on the glycosylated luminal domain and the cytoplasmic tail of gp210 in the nuclear pore complex (20), and reactivity has been associated with disease progression, active histologic inflammation, and progression to transplantation(21). Sp-100 is a nuclear body protein that consists of three major autoantigenic domains recognized by anti-sp100 sera, and yields a multinuclear dot immunofluorescence pattern(22). Nearly three quarters of PBC patients with prior urinary tract infections (UTI) test positive for anti-sp100 antibodies suggesting that bacteria or their by-products may have a role in auto-antibody formation(23). Anti-centromere(ACA) ANAs occur in up to 10% of patients with PBC, independent of limited scleroderma(24), and these patients may tend towards a portal hypertensive phenotype (25), though this remains controversial(26).

Novel Autoantibodies

Two novel PBC auto-antigens, namely kelch-like 12 (KLHL12) and hexokinase 1 (HK1) have recently been identified using a hypothesis-free proteomics strategy aimed at autoantigen discovery(27, 28). KLHL12 is a nuclear protein essential in collagen export and regulates protein ubiquitination(29). HK1 resides on the outer mitochondrial membrane and catalyzes phosphorylation of glucose to glucose-6-phosphate, maintains mitochondrial homeostasis, and modulates cellular susceptibility to apoptosis(30). Whether dysfunction of HK1 leads to mitochondrial dysfunction, loss of immune tolerance, and cell death remains an active area of investigation(31).

BILIARY EPITHELIAL CELLS

BECs line the biliary tree and participate in bile formation via apical and basolateral transmembrane channels and exchangers(32). Endogenous and exogenous stimuli including micro-organisms, xenobiotics and drugs activate BECs and modulate the inflammatory and proliferative response to injury and repair(33). Toll-like receptors (TLRs) respond to bacterial products including pathogen associated molecular patterns (PAMPs) and TLRs 1 through 5 expressed on BECs and are upregulated in PBC (34). Expression of Major Histocompatibility Complex (MHC) II, CD80 and CD86 allow BECs to serve as antigen presenting cells (APCs) and provide costimulatory signals for T cells, attesting to their active role in disease pathogenesis and propagation(35).

Biliary Bicarbonate Umbrella

AE2 is the major $Cl^-/HCO3^-$ exchanger expressed on BECs and regulates intracellular pH and biliary $HCO3^-$ secretion resulting in a bicarbonate rich "umbrella" on the apical surface of cholangiocytes, which protects BECs from toxic hydrophobic bile acids(36). Defective AE2 function facilitates acidification of bile salts rendering them hydrophobic and able to cross the plasma membrane leading to cellular apoptosis. Downregulation of AE2 leads to and alkaline intracellular environment, sensed by soluble adenylyl cyclase (sAC), a conserved bicarbonate sensor that sensitizes cells to apoptosis(37, 38). Consistent with this mechanism, AE2 deficient H69 cholangiocytes demonstrate increased sAC mRNA and protein, and inhibition of sAC inhibits bile salt induced apoptosis(39). Recent work suggests that miR-506-3p may target the 3' untranslated region (3'UTR) and downregulate AE2 in PBC with *in vitro* findings demonstrating reduction in AE2 expression, reduced $Cl^-/HCO3^-$ exchange activity, as well as mitochondrial dysfunction, PDC-E2 overexpression and sensitization of cholangiocytes to bile salt induced apoptosis(40). Futher to this, an AE 2 $a,b^{-/-}$ mouse model was developed that demonstrated enhanced production of IL-12 p70 and IFN-γ, expanded $CD8^+$ T cells, downregulated T regulatory cells (Tregs), AMAs and histologic evidence of mild to severe portal inflammation(41).

Biliary Injury, Apoptosis, and Senescence

Cholangiocytes are the primary epithelial source of TNF-α, a pro-inflammatory signal that can both promote apoptosis via the caspase cascade, and activate survival pathways via the nuclear factor κB pathway(10). TNF-α (in combination with IL-1, IL-6 and IFN-γ) inhibits cyclic-AMP (cAMP) mediated ductal secretion, while the IκB kinase (IKK)/NF-κB signalling pathway regulates inflammatory responses and protects against oxidative and cytokine mediated damage and death(42).

Usually, apoptotic cells modify mitochondrial PDC-E2 through covalent binding of glutathione. In PBC, this modification does not occur and the antigenic lysine-lipoyl epitope remains immunologically intact within an apoptotic bleb(17). This apotope is recognized by circulating AMAs and the resultant antigen-antibody complex subsequently stimulates the immune system resulting in widespread immune activation leading to apoptosis of neighbouring cells(43). As APCs themselves, cholangiocytes have been implicated in loss of tolerance via impaired phagocytic clearance of apoptopes(44). This aberrant mechanism of protein degradation may be responsible for the BEC directed injury in PBC.

Senescence, the state of permanent cell cycle arrest is a protective mechanism to remove damaged cells from the local environment(45); though senescent cells accumulate in PBC (46). Furthermore, rather than being replaced by normal cells, senescent cholangiocytes transition to a “senescence associated secretory phenotype” characterized by secretion of diverse cytokines (IL-6, IL-1), chemokines (CX_3CL1, CXCL8, and CCL2), growth factors, and matrix

metalloproteinases (MMPs) that recruit additional immune cells and act in the process or remodelling and repair(47).

HEPATIC IMMUNOREGULATION IN PBC

Innate Immune Response

The innate immune response is not independently sufficient to cause a breakdown in tolerance, though the presence of granulomatous inflammation, polyclonal IgM production, increased natural killer (NK) cells, and cytokine responses emphasize its importance in the pathogenesis of disease(48). Microbial PAMPs, lipopolysaccharides (LPS) and lipotechoic acids in bile can bind to cell surface TLRs on BECs leading to biliary injury via the pro-inflammatory NF-κB pathway and chemokine release (IL-8 and CX3CL1)(34). CX3CL1 attracts $CD8^+$ and $CD4^+$ T cells, is upregulated in injured bile ducts and T cells with its cognate receptor are found in portal tracts of subjects with PBC(49).

NKT cells are innate effector cells regulated by antigen presentation by CD1d(50); and an increased frequency of CD1d restricted NKT cells is seen in PBC with a higher proportion in the liver compared to peripheral blood(10). In addition, increased cytotoxic activity and perforin expression by NK cells has also been described in PBC(51). In another potential link between the innate and adaptive arms, Shimoda *et al*. demonstrate that at high NK/BEC ratios, NK cells attack BEC resulting in autoantigen release, which in the presence of APCs activate autoreactive T cells(52); whereas, at low NK/BEC ratios, BECs experience indirect injury, though IFN-γ secreted from NK cells which induces HLA II expression on BECs which are subsequently targeted by autoreactive $CD4^+$ T cells(52).

Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T cells characterized by expression of a semi-invariant T cell receptor (TCR) chain (Vα7.2-Jα33) and restricted to MHC class I that can be activated independently or by microbial products to produce pro-inflammatory cytokines including IFN-γ, TNF-α, and IL-17(53). Recently, Setsu *et al*. reported significantly reduced numbers of MAIT cells in the blood and liver of PBC patients compared to controls with circulating MAIT cells demonstrating impaired production of cytokines (particularly TNF-α). Furthermore, despite biochemical response to UDCA, frequencies of MAIT cells increased but did not normalize, suggesting that ongoing biliary injury and progressive disease may occur despite biochemical improvement(54, 55).

Adaptive Immune Response

The role of the cellular immune response in PBC is emphasized by the presence of highly specific AMAs and heavy infiltration of $CD4^+$ and $CD8^+$ T cells in the portal tracts of patients with disease(13, 15). Independent of detectable autoantibody, antigen specific $CD4^+$ and $CD8^+$ T cells are enriched 100-fold and 10-fold, respectively, in the liver as compared to peripheral blood(16). Several other T cell subpopulations have also been implicated, including the pro-inflammatory, pro-fibrotic $CD4^+$ T helper (Th) 17 subclass(56), regulatory T cells (Treg) which modulate immunity and promote self-tolerance via suppression of inappropriate immune activation(57), and follicular helper T cells (Tfh) which facilitate B cell differentiation and antibody production in germinal centers(58).

The portal tracts in PBC are rich in chemokines including CXCL10, CXCL9, and CX3CL1 which recruit $CD4^+$ and $CD8^+$ T cells bearing their cognate receptors(59). While both CD4 and CD8 T cells recognize similar sequences within the 2-OADC enzyme complexes, $CD8^+$ T cells likely play an important role in cell death of targeted cholangiocytes(60, 61). An increase in Th17 cells

within the liver of patients with PBC as compared to controls has also been reported, with hepatic Th17 infiltration increasing, but circulating Th17 cytokines decreasing with progression of fibrosis(62). Disease progression is associated with skewing from a Th1 to Th17(63) predominant cytokine profile which congregate around damaged cholangiocytes and can further promote injury(60).

Tregs ($CD4^+CD25^+FoxP3^+$) T cells, control excessive immune responses by modulation of APC maturation, metabolic disruption, and secretion of anti-inflammatory cytokines(64). Anti-inflammatory cytokines such as IL-10 and TGF-β as well as PD-L1 and CTLA-4 contribute to the immunosuppressive function of Tregs which are converted in the liver from naïve or effector $CD4^+$ T cells. A relative reduction in the number of Tregs in peripheral blood has been observed in PBC patients compared to patients with controls(62) and intrahepatic Tregs are also reduced in PBC patients with the intrahepatic CD8/Treg ratio being higher in those with PBC compared to patients without bile duct damage(65). In the dnTGFβRII mouse model of PBC, Tanaka *et al*. demonstrate that murine $CD4^+FoxP3^+$ T regs possess weaker suppressive function than wild type Tregs(66).

Follicular helper T cells (Tfh) localize to germinal centres within lymphoid follicles and provide requisite B cell support to produce highly antigen specific antibodies and generate B cell memory(67, 68). T follicular regulatory T (Tfr) have been recently described, which localize to germinal centres, and analogous to Tregs, suppress the humoral immune response elicited by Tfh cells(69). Wang *et al*. demonstrated increased number and function of peripheral Tfh in PBC and a decline in in frequency of Tfh associated with treatment response to UDCA(67). In an extension of this work, Zheng *et al*. showed significant reduction in circulating Tfr cells and the Tfr/Tfh ratio in PBC which negatively correlated with serum IgM levels(69).

GENETIC PREDISPOSITION

The importance of genetic risk in development of disease is highlighted by several lines of evidence that demonstrate high concordance of disease in monozygotic twins, an increased prevalence in first through fifth degree relatives (FDRs) of affected probands(70), a sibling relative risk of 10.5, and a high prevalence of AMAs in FDRs compared to population controls(71-73). While genetics play an important role, it is likely that allelic variants are not deterministic, but modulate important biologic processes that lead to disease. While significant advances have been made in our understanding of the genetic architecture of PBC, the functional consequences of identified variants and their relevance to important biologic pathways remains undefined.

Human Leukocyte Antigen (HLA)

HLA genes are located in the highly polymorphic MHC region, encode molecules responsible for antigen presentation, and are essential in establishing immune tolerance(6). Candidate gene and genome wide association studies (GWAS) have demonstrated robust PBC-specific associations at the DRB1, DQA1, and DQB1 loci with many variants mapping to antigen binding regions of MHC molecules possibly leading to defective antigen presentation(74). The HLA DRB1*08 family is associated with disease, specifically DRB1*0801 in European and North American cohorts, and DRB1*0803 among the Japanese, giving insight into ethnic variability of disease predisposition(75, 76). Furthermore, HLA DRB1*11 and HLA DRB1*13 were protective against disease in European cohorts, whereas DQB1*06:04 and DQB1*03:01 conferred a reduced risk of disease among the Japanese(77, 78). GWAS data have suggested that HLA subtypes may be associated with distinct immunologic phenotypes, with single nucleotide polymorphisms (SNPs) at the HLA-DPB1 locus associated with anti-sp100 positivity among a

North American and European cohort, and HLA-DRB1*0405 and HLA-DRB1*0803 being associated with anti-gp210 and anticentomere antibodies, respectively, in a Japanese study(79, 80). Notably, while the strongest statistical associations in GWAS have consistently been at the HLA locus, these risk alleles are present in less than 15% of patients and despite statistically robust associations, they are lower
than seen in many other autoimmune diseases(81).

Non-HLA Risk Loci

In the last several years, large scale GWAS efforts have identified dozens of SNPs associated with PBC, and have highlighted several important pathways in antigen presentation, lymphoid differentiation, and B cell function as contributing to disease(5). The first GWAS from Canada identified SNPs at HLA, *IL12A and IL12RB2* as significant associations with PBC and highlighted the importance of the IL-12 signalling pathway in this disease(82). Another effort including Italian and Canadian subjects confirmed associations from the initial GWAS, and also identified loci that map to regions containing *IRF5-TNPO3, IKZF3,* and *SPIB*, each of which has an immune-regulatory function(82, 83). A large GWAS and meta-analysis from the UK subsequently identified 12 additional loci associated with PBC and emphasized the role of the NF-κB pathway, T cell differentiation, TLR, and TNF signalling, with SNPs mapping to *STAT4, DENDD1B, CD80, IL7R, CXCR5, TNFRSF1A, CLEC16A, and NFKB1* as significant associations(84).

Ethnic differences in genetic susceptibility were highlighted by a Japanese GWAS that implicated *TNFSF15* and *POU2AF1* as risk loci, but failed to identify associations with the majority of other non-HLA variants including *IL12A and IL12RB2* found in predominantly Caucasian populations(85). A recent GWAS of the Han Chinese population replicated 14 previously reported risk loci including *IL12A* but identified six novel variants at *IL21, IL21R,*

CD28/CTLA4/ICOS, CD58, ARID3A and IL15 associated with disease(86). Variants at *IL21 and IL21R* were strongly associated with disease and aberrant expression of IL21 and IL21R was demonstrated on immunohistochemical analysis of livers of PBC patients. Interestingly, recent studies have reported increased IL21 expression from Tfh cells in PBC patients, which have a role in mediating B cell maturation, differentiation, and antibody production, as discussed earlier(67).

Cordell *et al*. performed an meta-analysis and pathway analysis in an effort to identify pathways most relevant to disease. In addition to identifying 6 novel PBC risk loci, several immunoregulatory pathways were highlighted including IL-12, IL-27, and JAK-STAT signalling, even after adjusting for bias associated with their HLA contribution(87). Hitomi *et al*. performed high density mapping, *in silico* and *in vitro* analysis to identify a functional variant at the 17q12-21 risk locus, and identified rs12946510 as the SNP influencing gene expression via alteration of the Forkhead box protein O1 (FOXO1) binding motif(88). Disruption of the enhancer regions of *ORMDL3* and *GSDMB* as a result of this variant translated into lower gene expression levels, suggesting a functional relevance of this susceptibility locus. Collectively, this body of evidence has implicated many genes in PBC, yet more than 80% of the heritability of disease remains unexplained, possibly related to the contribution of rare variation with strong biologic effect, non-SNP structural changes including epigenetic modification, or gene-gene and gene-environment interactions(89). That said, GWAS have certainly highlighted the role of immune dysregulation in PBC and key among these is the IL-12 signalling axis.

IL-12 in PBC

IL-12 is heavily involved in development of T_H1 responses, a key feature of autoreactivity in PBC(90). The IL-12 cytokine family (IL-12, IL-23, IL-27, IL-35) is associated with bidirectional immune regulation and *IL12A* and *IL12RB2* are the strongest non-HLA risk loci associated with

PBC in large scale genetic efforts(91). Functional IL-12 interacts with its $CD4^+$ T cell surface receptor to activate a T_H1 response via JAK-STAT signalling; and genes regulating downstream components of this pathway including *TYK2* and *STAT4* have been associated with disease in GWAS(92). Engagement of IL-12 with its receptor also modulates the immune response via IFN-γ which inhibits proliferation of pro-inflammatory T_H17 cells by IL-23, a negative regulator which blocks T_H1 and T_H17 development and supports proliferation of Tregs(93).

GWAS have also implicated pathways upstream of IL-12 in PBC. Interferon regulatory factor 5 (protein product of *IRF5*) interacts with NF-κB to activate T_H1 cytokines, including IL-12, and transcription factors encoded by *IRF8* bind to *IL12* promotors to regulate IL-12 and IFN-γ production(94). Despite absence of statistical associations with *IL12* loci in the Japanese GWAS, genes including *TNFSF15* were implicated and their protein products interact with death receptor 3 to promote T_H1 and T_H17 expansion and interact with IL-12 to promote IFN-γ production(85). With this data in hand, Ustekinumab, a monoclonal antibody targeting the p40 subunit shared by IL-23 and IL-12 was an attractive therapeutic target in PBC. Unfortunately, in a proof of concept phase II study, while nearly half of patients had a decline in ALP by more than 20%, no patient reached the predefined primary endpoint of the study(95).

EPIGENETICS and MICRORNA

MicroRNAs in PBC

MicroRNAs (miRNA) are small RNA molecules that are important in post transcriptional regulation of gene expression and modulate diverse biologic processes(96). Differential expression 35 hepatic miRNAs in PBC has been shown with targets predicted to affect cell proliferation, injury, and cell death(96). An association between miR-506 and AE2 regulation is an intriguing potential pathophysiological link in PBC in light of the integral role AE2 plays in

maintenance of the protective bicarbonate rich layer on the apical surface of cholangiocytes(40). In fact, miR-506 is increased in PBC livers and upregulated in intrahepatic bile ducts in PBC compared to other chronic cholestatic liver disease. Over expression of miR-506 in PBC is associated with decreased AE2 expression and activity in human cholangiocytes (likely via binding to the 3'UTR of AE2 mRNA) and improvement in AE2 activity is seen after transfection with anti-miR-506(40). Futheremore, pro-inflammatory cytokines stimulate miR-506 gene expression in cholangiocytes and this overexpression inhibits AE2 leading to widespread dysregulation of multiple biologic processes particularity related to mitochondrial metabolism(97). Type III inositol 1,4,5-triphosphate receptor (InsP3R3) is a major intracellular calcium release channel located in the endoplasmic reticulum of cholangiocytes and also promotes biliary bicarbonate secretion, and miR-506 has also been shown to be a regulator of this gene (*$InsP_3R_3$*)(98). Interestingly, miR-506 is an x-linked microRNA localized at Xq27.3(99); that aberrant epigenetic X-inactivation and resultant miR-506 upregulation could relate to the female predominance of disease, remains an intriguing hypothesis.

MiRNAs have also been linked to immune. MiR-92a is downregulated in PBC, is inversely associated with Th17 populations, and is co-expressed with IL-17A in PBMCs suggesting its potential involvement in upregulation of this cell subset(100). Downregulation of microRNAs related to $CD4^+$ T cell receptor signalling have also been implicated, in particular those that target *N-Ras*, which has an important role in T cell activation(101). Microarray and quantitative real-time polymerase chain reaction (qRT-PCR) analysis have shown decreased miR-425 associated with induction of proinflammatory IL-2 and INF-γ via N-Ras mediated upregulation of TCR signalling(101).

Sex Chromosomes and Epigenetics

Incomplete concordance of PBC between monozygotic (MZ) twins emphasizes that factors in addition to genetics must play a role in disease onset, and epigenetic mechanisms including DNA methylation, histone modification, and non-coding RNA's are being investigated for their potential role(102). Abnormal methylation patterns in PBC were reported in a unique cohort of 3 discordant MZ twin pairs and eight sister pairs of similar age and demonstrated 60 and 14 differentially methylated regions in MZ and sister pairs, respectively, with hypermethylation observed in the PBC proband and 85% of methylation regions localized to the X chromosome in MZ pairs(103). Despite absence of significant associations in GWAS, the CD40-CD40L system has long been postulated to be relevant in PBC as it is X-linked, essential in T cell priming, immunoglobulin class switching and peripheral B cell tolerance, and has been associated with elevated IgM levels in immunodeficiency syndromes associated with mutations of the CD40L gene(104); indeed, Lleo *et al*. demonstrated reduced DNA methylation of the CD40L promotor in $CD4^+$ cells in PBC patients which was associated with increased CD40L expression(105). More recently, the X chromosome methylation profile of CD4, CD8, and CD14 cells from PBC patients were reported (106), and hypermethylation of FUNDC2 in $CD8^+$ cells and hypomethylation of CXCR3 in $CD4^+$ cells was associated with increased CXCR3 expression, intriguing in light of the integral role of CXCR3 in in leukocyte trafficking(106). The complexity of epigenetic regulation in PBC beyond promotor methylation status was emphasized by a study of 3 discordant MZ twins which showed consistent downregulation of *CLIC2* and *PIN4* genes in affected twins, but for both genes, promotor methylation was partial, variable, and did not predict transcript levels or X chromosome inactivation(107).

ENVIRONMENTAL RISK

Variable risk in families, regional differences in prevalence, and reproducible epidemiologic associations between disease and environmental agents suggest that exposures are likely requisite for PBC to develop. Molecular mimicry is presumed to be the mechanism whereby T and/or B cells primed against cross-reactive antigenic mimics lead to loss of self-tolerance to the mitochondrial PDC-E2 autoantigen (108). While a variety of exposures have been described, bacterial infections, xenobiotics, and smoking history, have been the most robustly studied (Figure 3)(109).

Large scale studies of patients with PBC report higher rates of incident and recurrent UTI and demonstrate an increased frequency of bacteruria compared to controls(110, 111). On a molecular level, protein motifs are strongly conserved among species and in fact, human and *E.coli* PDC-E2 share the molecular sequence (ExDK) essential for recognition by autoreactive PDC-E2 specific $CD4^+$ T cells. Furthermore, sera from patients with PBC react with both human and *E.coli* PDC-E2(112). *Novosphingobium aromaticus (N. aro)*, a xenobiotic metabolizing bacteria identified through a search for PDC-E2 homologues, contains lipoylated proteins that are up to 1000-fold more reactive with PBC patient sera than even *E.coli*, making this pathogen another potential etiologic trigger(113). Associations between PBC and several other pathogens including *Helicobacter pylori(114), Chlamydia pneumoniae(115), Mycobacterium gonordae(116),* and *Lactobacillus delbruekii* have been reported, though many of these associated have not been reproducible(117).

Xenobiotics, or foreign chemicals that can alter self proteins, have also been implicated in PBC. Epidemiologic associations between use of nail polish, smoking history and residence adjacent to toxic waste sites are supportive of their role (109). 2-octynoic acid (2-OA) is present in cosmetics and food additives and is a putative candidate showing enhanced reactivity with PBC sera in a structure-activity relationship analyses(118). The role of 2-OA as a relevant xenobiotic

was further studied by immunization of 2-OA-bovine serum albumin complexes into C57BL/6 mice and, indeed, high titer AMAs, increased $CD8^+$ T cell hepatic infiltration, and upregulation of TNF-α and IFN-γ was observed(119). Similarly, in the NOD.1101 model, in addition to high titer AMAs, portal granulomas typical of human PBC were noted(120). 2-nonyamide has an optimal chemical structure for xenobiotic modification of PDC-E2 resulting in enhanced reactivity with AMA positive sera(121). An association between PBC and exposure to volatile organic compounds (including benzene) is based on studies demonstrating clustering of cases adjacent to open-air toxic waste sites(122) and higher rates of disease among subjects exposed to cigarette smoke, including through indirect exposures(123, 124). In fact, halogenated benzene can mimic PDC-E2 and reacts with AMA positive PBC sera(118).

SUMMARY

PBC is a classic autoimmune disease, characterized by auto-reactivity against a well described and highly conserved mitochondrial antigen. In a genetically susceptible host, exposure to an environmental mimic of PDC-E2 may incite a promiscuous immune response targeted to the biliary epithelia. Biliary injury and an alkaline intracellular environment attributed to defective AE2 function may sensitize BECs to apoptosis, leaving an immunogenic epitope in tact within an apoptotic bleb and resulting in focused biliary injury despite an otherwise ubiquitous antigen. A resultant multi-lineage response involving both innate and adaptive immune responses may further propagate biliary damage and is aggravated by an imbalance between intrahepatic and peripheral effector and regulatory cells. Genome wide efforts have failed to identify disease specific pathogenic variants but have highlighted several important immune regulatory pathways, key among them being IL-12 signalling. Moving forward, expanding on these insights to further dissect the molecular pathogenesis of this complex disease remains essential with the aim to develop novel therapies targeted and the most relevant biologic pathways.

Research Agenda

- Future genetic efforts should concentrate on determining the functional biologic relevance of identified variants and use technologies that decipher important cellular regulatory networks
- The role of environmental triggers in contributing to disease onset needs to be thoroughly investigated
- Collection of detailed clinical, immunologic, and histologic data from well characterized cohorts will enable molecular characterization of clinically relevant sub-phenotypes of disease

Practice Points

- Disease development requires a permissive genetic background and exposure to an as yet undefined environmental trigger
- Aberrant apoptotic pathways produce an immunogenic apoptotic bleb that likely confers liver specific injury despite an otherwise ubiquitous autoantigen
- An imbalance between effector and regulatory immune activity results in self-perpetuating biliary injury which manifests clinically as progressive liver disease

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Figure 1.

Figure 1. Pathophysiology of PBC.

Exposure to an microbial or environmental mimic of PDC-E2 leads to a multi-lineage immune response targeted to the biliary epithelial cells. Biliary injury and defective AE2 leads to disruption of the bicarbonate biliary umbrella, sensitizing biliary epithelium to apoptosis. Aberrant apoptotic pathways due to lack of covalent binding of glutathione leaves the immunogenic epitope in tact within an apoptotic bleb resulting in perpetual and focused biliary epithelial injury. A promiscuous immune response involving the innate and adaptive arms further propagates biliary injury and is characterized by an imbalance between effector and regulatory immunity ultimately resulting in continuous inflammation leading to cholestasis and biliary fibrosis. *Illustration By: Qingyang Chen*

Figure 2. Conserved Lysine-Lipoic Acid motif.

PDC-E2 and E2 subunits of other mitochondrial autoantigens contain an essential lysine residue within the lipoyl domain to which lipoic acid is covalently attached. This lipoic-lysine bond at position 173 is highly conserved across species and is necessary for antigen recognition.

Illustration By: Qingyang Chen

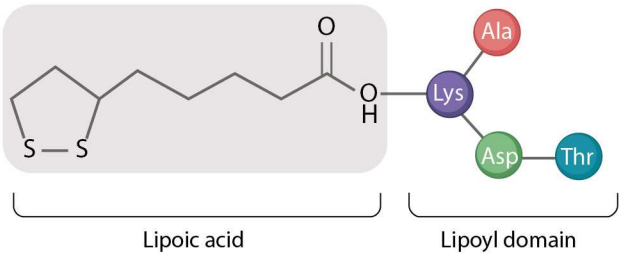
Figure 3. Aggregate factors result in disease manifestation

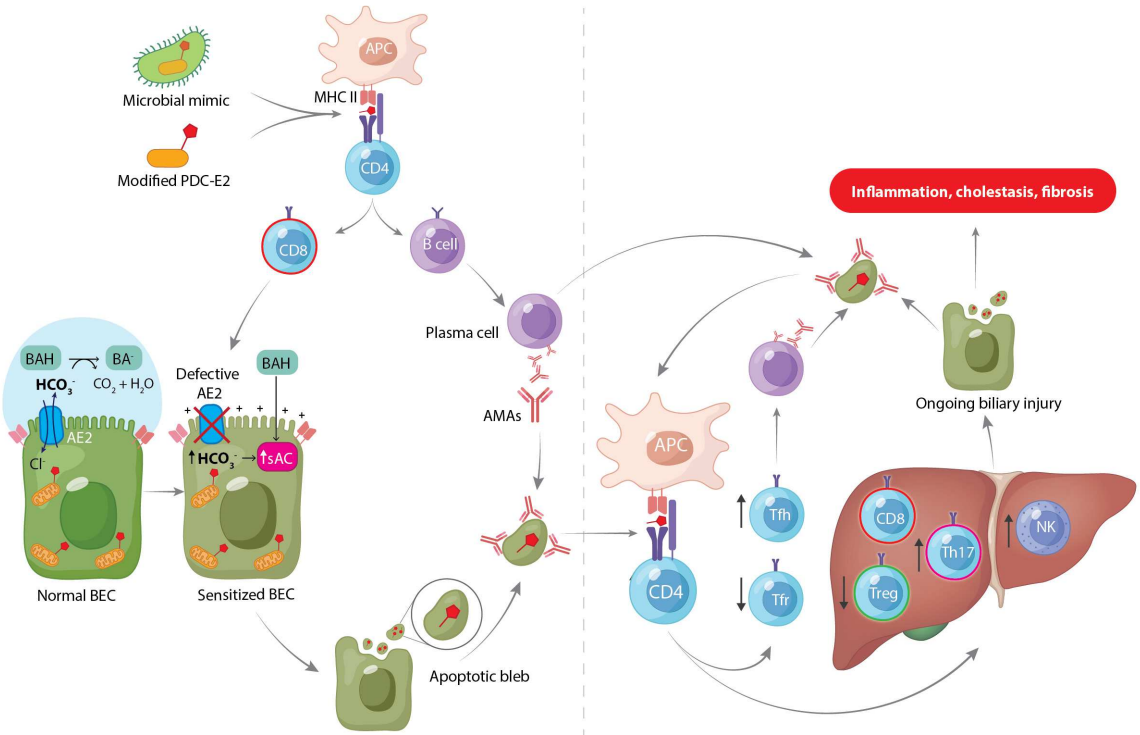
Clinical manifestation of disease is a result of an aggregate of factors that result in pathology. It is likely that a permissive genetic background, requisite environmental trigger and non structural genetic influences including epigenetic regulation are required within a host that elicits a dysregulated immune response culminating in biliary injury and clinical disease. *Illustration By: Qingyang Chen*

Table 1. Autoantigens and autoantibodies in Primary Biliary Cholangitis (PBC)*

Autoantibodies	Location	IF pattern	Target	PREVALENCE AMA positive (%)	AMA negative (%)
Anti-mitochondrial antibody	inner mitochondrial membrane		PDC-E2		
			OGDC-E2		
			BCOADC-E2		
			E3BP		
Anti-nuclear antibodies				47–48	68–85
	nuclear pore complex	nuclear rim	gp-210	16–18	15–45
			p62		
	nuclear body	multinuclear dot	sp100	24–31	38–54
			PML		
Anti-centromere antibodies	centromere	anti-centromere	CENP	14–20	14–23
Anti-kelch	nuclear protein		KLHL12	42	35
Anti-hexokinase	outer mitochondrial membrane		HK1	53	22

*adapted from reference 10 Abbreviations: BCOADC, branched-chain 2-oxo-acid dehydrogenase complex; CENP, centromere protein; E3BP, E3-binding protein; gp210, glycoprotein 210; OGDC; HK1, hexokinase 1; KLHL12, kelch like 12; oxoglutarate dehydrogenase complex; sp100, nuclear body speckled 100 kDa; PDC, pyruvate dehydrogenase complex; PML, promyelocytic leukemia; 2-OADC, 2-oxo-acid dehydrogenase complex.





A) Trigger, loss of tolerance, biliary injury and apoptosis

B) Multilineage immune dysregulation